

**Identification of virulence-associated genes in *Mycobacterium avium* subsp. *paratuberculosis* by mutant-library construction**

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## Final report for BARD grant IS-4905-16 R

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Dr. Daniel Barkan (Hebrew University, Israel)

Dr. Yung-Fu Chang (Cornell University, NY)

### **Abstract:**

**Background:** *Mycobacterium avium paratuberculosis* (MAP) is a slow growing bacterium causing severe disease in cattle, resulting in substantial economic losses to the cattle industry. The project concerns with identification of bacterial genes essential for viability, pathogenesis, survival in macrophages, and virulence in animals. The original Aim was the engineering of a novel transposon delivery and selection system, creation of a saturated transposon mutant library, analyzing this library by modern sequencing methods, and identifying genes essential for viability.

**Methods:** We constructed a novel transposon/transposase system, based on a double antibiotic selection, placed it in a phage, infected MAP bacteria, isolated mutants that got the transposon, analyzed their DNA by deep sequencing on an illumine-platform, and built a bioinformatics algorithm to pin point the affected gene. This was repeated for several growth conditions, allowing identification of virulence genes.

**Results:** We completed the design and creation of a novel transposon tool; we showed it works; we used it to create a library in *M. abscessus*, leading to highly intriguing and novel discoveries in the genetics of this bacterium; we used it to create a library in MAP; we validated the bioinformatics strategy we intend to use; and are now in the midst of building the computer script for the analysis of the library.

**Conclusions:** We completed Aim 1 of the original grant, and are now ready to submit a full proposal for continuation of research. Also, we performed additional research on drug resistance of MAP and *M. marinum*, and submitted the results for publication (under review now).

## Summary Sheet

### Publication Summary

PubType	IS only	Joint	US only
Submitted	0	1	0

### Training Summary

Trainee Type	Last Name	First Name	Institution	Country
Postdoctoral Fellow	Lee	Jen-Jie	Cornell	USA
M.Sc. Student	Grosfeld	Tatyana	Hebrew U	Israel
Ph.D. Student	Bar Oz	Michal	Hebrew U	Israel

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### **Contribution of collaboration:**

The transposon system was developed in Israel.

The actual infection of MAP bacteria by the transposon (the creation of the bacterial library) was done in the USA, where there is a lot of experience with growth of MAP in-vitro. A parallel library was prepared in MAP mutants that are deleted in the *leuD* gene (a mutant prepared previously by Dr. Chang's laboratory).

The Bacterial library was sent to Israel, where it was grown in several conditions, and the genomic library prepared.

The sequencing was done as out-sourcing in the University of Chicago.

The bio-informatic analysis is presently done in Israel.

The imminent infection experiments (where macrophages and then live cows will be infected by the mutant library) will all be done in the USA in Dr. Chang's lab.

The bioinformatics analysis will be done in Israel, by Dr. Barkan.

It is therefore obvious the project could not have been done – definitely not in the full extent – if it was not for the collaboration of both laboratories.

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Dr. Daniel Barkan (Hebrew University, Israel)

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#### **Background, original, and modified plan:**

*Mycobacterium avium paratuberculosis* (MAP) is a slow growing bacterium causing severe disease in cattle, resulting in substantial economic losses to the cattle industry. It is also sometimes implicated in the pathogenesis of human Inflammatory Bowel Disease (Crohn's colitis). The project concerns with identification of genes essential for viability, pathogenesis, survival in macrophages, and virulence in whole animals of this pathogen. The original three stages (Aims) were:

1. Engineering a novel transposon delivery and selection system, creation of a saturated transposon mutant library, analyzing this library by modern sequencing methods, and identifying genes essential for viability.
2. Identifying genes essential for conditions relevant for pathogenesis (high acid, radicals, detergent stress, starvation, antibiotic stress) using *in-vitro* selection.
3. Performing large-animal experiments, in which the library will be used to infect calves in the ligated-loop model, examining these genes in a whole organism model.

BARD funding was only provided for one year, and then extended for an additional year, to allow completion of Aim 1 (which must be completed to continue the study). We are happy and proud to say **we now completed Aim 1, and we intend to apply for renewal of the grant in the next available application date.**

#### **Results:**

##### The creation of a system to introduce the transposon/transposase system into mycobacteria, and specifically MAP:

A transposon is a genetic element, that can insert itself into a random area in the target genome (in this case – the bacterial genome), thus interrupting a gene. In mycobacteria, the transposon/transposase system is introduced into the cell by a phage (a virus that infects bacteria). We created a phage with a novel transposon, called phDB33: unlike other transposons, it has two selection markers (resistance to zeocin and to kanamycin – thus making the selection very stringent, and virtually no background), and it is engineered to enable the use of a highly novel transposon-insertion site sequencing method, allowing high throughput identification of all the different mutants in a mutant library. The Inverted Repeats (IR) of the transposon were specifically constructed to contain the recognition site for the restriction enzyme MmeI, which digests 18bp laterally to the IR (into the adjacent genome).

Of note, we completed the construction of phDB33 in the middle of 2018. Recently, we completed the construction of another phage with a modified transposon: phDB37. The transposon in phDB37 confers resistance to zeocin and hygromycin (thus, also zero background, like phDB33), but we also introduced several point mutations in the sequence, to make the insertion-site identification even easier. Although we

made substantial progress in the MAP library, in the future we will use phDB37 for additional library creations in other mycobacteria, such as *M. abscessus*, and possibly an additional library in MAP.

#### Testing the capability of the phage to infect and introduce the transposon in random genomic sites:

To test the ability of phDB33 to infect and introduce the system into mycobacteria, we tested it in both *M. bovis* and in *M. abscessus*. Whereas a successful transposon library was created previously in *M. bovis*, no such library was made in *M. abscessus* (despite several attempts by others, using a different phage). We infected both bacteria by phDB33, and plated the bacteria on plates with kanamycin AND zeocin. We got multiple single colonies. In both cases, we picked random 10 colonies, and verified by PCR they indeed have the transposon inserted in the genome. To confirm the insertion was at random sites, we identified the insertion site in the 10 random clones, and indeed showed that it was a different site for each clone.

#### Identification of important genes in *Mycobacterium abscessus*:

*M. abscessus* is an emerging human pathogen. We used phDB33 to create a limited transposon library, picked 7 colonies with an altered colony morphology, and performed insertion site identification. Analyzing the results revealed a novel small non-coding RNA molecule that regulates a secretion system, indispensable for pathogenesis. This opened a whole new avenue of investigation, that is currently actively pursued in the lab with very interesting results. Although this was not an official Aim of our research (and grant), the discovery would not have been made without the transposition tool, designed for this grant.

#### Infecting MAP with phDB33 create a transposon mutant library:

To construct the actual library in MAP, we infected it with phDB33. The infecting phage enters the bacterium, thus delivering the transposon/transposase into it. Once in every 10,000 events, the transposon actually enters the bacterial genome, conferring resistance to zeocin and kanamycin. We performed the infection several times, each time collecting several thousands of mutants, altogether, we believe we collected ~30,000 mutants.

#### Preparation of the genomic library:

The pool of mutants, containing approximately 30,000 clones, was frozen in several vials. One vial was grown, genomic DNA was extracted, and digested by MmeI. The fragments were blunted (using a blunting enzyme), then re-ligated (by self-ligation), and a PCR reaction was performed. The primers are specific to the transposon, so that only fragments containing the transposon were amplified. The PCR product contains parts of the transposon as well as 12 base-pairs from the bacterial genome that are the exact location of the transposon insertion. The PCR products were sent for mass sequencing on an illumine-based platform (University of Chicago). Results were obtained in August 2019.

#### Identification of the insertion sites using bioinformatics analysis:

The sequencing results were first examined manually, and we confirmed the product indeed contains 12 base pairs from the genome, and this sequence allowed identification of the insertion site. With the help of Dr. Moran Gershoni, a bioinformatics expert in the Volcani Institute, we are now programming a computer code that would analyze all the 100,000 sequences, find the corresponding sequence in the annotated MAP K-10 genome, and produce a list of all the insertion sites. This will allow for: 1) determine the extent of the

library) how good it really is; 2) determine which genes are needed for viability of the bacteria, and which are dispensable. We expect to have this script ready and running in October 2019.

#### Identification of genes needed for antibiotic resistance and survival in starvation:

We grew the library in the presence of the sub-inhibitory concentrations of the antibiotic ciprofloxacin, that causes DNA damage. The surviving bacteria were collected, and a genomic library was prepared in exactly the same procedure as above. The genes will be analyzed with the same computer script (around October 2019). By comparing the list of genes needed for viability without selection, to the genes needed with selection, we will pinpoint exactly the genes needed for surviving ciprofloxacin-induced DNA damage.

A similar procedure is done with starvation: bacteria were put in PBS (no nutrients) for 2 weeks, then recovered in media, and genomic DNA analyzed. We will compare the genetic profile of the starvation-survivors with that of the preliminary library. As survival in starvation is a major ability of bacteria, that is needed for pathogenesis, this will identify genes indispensable for the bacteria to cause disease.

#### To summarize this part:

We completed the design and creation of a novel transposon tool; we showed it works; we used it to create a library in *M. abscessus*, leading to highly intriguing and novel discoveries in the genetics of this bacterium; we used it to create a library in MAP; we validated the bioinformatics strategy we intend to use; and are now in the midst of building the computer script for the analysis of the library.

**We therefore completed the original Aim 1**, and are ready to proceed to Aims 2 and 3. We will apply for continued funding from BARD in the nearest possible application round. We expect to submit our results for publication in 4 months from now.

#### Additional joint research, leading to publications:

As part of our collaboration, we researched the biochemical basis of isoniazid (an important medication) resistance in the fish pathogen *M. marinum* and in MAP. In *M. tuberculosis*, where the medication is highly active, the drug is activated into its active form by a bacterial enzyme called KatG. We showed that the KatG of *M. marinum* and of MAP do not activate isoniazid. When we introduced KatG from *M. tuberculosis* into them, they became sensitive to the drug. We performed biochemical analysis of the KatG proteins from all these bacteria, and showed the KatG of the resistant ones does not bind to isoniazid. Our findings are important both for treating isoniazid-resistant tuberculosis, and for possible development of drugs against MAP and *M. marinum*.

These findings **were submitted** for publication, and are currently **under review**:

Tali H. Reingewertz<sup>1</sup>, Tom Meyer<sup>1</sup>, Yung-Fu Chang<sup>3</sup>, Marcel A. Behr<sup>2</sup>, Daniel Barkan<sup>1,\*</sup>: **Differential sensitivity of mycobacteria to isoniazid is related to differences in KatG-mediated enzymatic activation of the drug.**

Publications for Project IS-4905-16 R

Stat us	Type	Authors	Title	Journal	Vol:pg Year	Cou n
Submitted	Reviewed	Reingewertz, Meyer, Chang, Behr, Barkan	Differential sensitivity of mycobacteria to isoniazid is related to differences in KatG- mediated enzymatic activation of the drug.		:	Joint